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1 **Genetic structure of natterjack toad (*Epidalea calamita*) populations in Flanders,**
2 **Belgium, and its implications for conservation.**

3 Joke Maes¹, Arend Raoul Van Oosten^{1,2}, Natalie Van Houtte¹, Erik Matthysen¹.

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5 ¹Evolutionary Ecology Group, Department of Biology, University of Antwerp,
6 Groenenborgerlaan 171, B-2020 Antwerp, Belgium

7 ² Department of Animal Ecology, VU University Amsterdam, De Boelelaan 1085, 1081
8 HV Amsterdam, The Netherlands

9

10 Correspondence to J. Maes. E-mail: joke.maes@uantwerpen.be.

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Abstract

Unique evolutionary potential could be lost when a population goes extinct or when individuals are translocated to other existing populations. Therefore, in order to identify priorities and to predict the efficiency and consequences of conservation actions, information is needed on the genetic structure of natural populations. In the urbanized and diverse landscapes of Flanders, Belgium, natterjack toad (*Epidalea calamita*) populations have been declining over the last decades. Therefore, this species is subjected to a wide range of different types of conservation measures (e.g. habitat management, corridor development, translocations). However, more information is needed on its genetic population structure. In this study, we sampled egg clutches from six populations and studied their genetic structure with six microsatellite markers. In total, 184 samples from 99 different egg strings were genotyped. Observed heterozygosity was generally high, even for the small and isolated populations (overall mean $H_O = 0.43$). The weak clustering by the Bayesian analyses (STRUCTURE, Adegnet and BAPS) does not allow us to make strong conclusions on the population structure. However, the significant Φ_{ST} values between the populations underline the importance of genetic information when conservation priorities are discussed. Unique evolutionary potential could be lost when one or more natterjack toad populations would go extinct, and translocation of individuals to other existing populations should be considered with caution.

Key words: *Epidalea calamita*, conservation, genetic differentiation, microsatellites, population genetics.

Introduction

Habitat loss and fragmentation are widely recognized as major threats to biodiversity on a regional as well as on a global scale (Harrison and Bruna, 1999; Fahrig, 2003; Henle et al., 2004). A strong linkage between scientific information and landscape policy formulation is crucial for a successful and efficient biodiversity conservation strategy (Messmer et al., 2001; Tabarelli and Gascon, 2005). In a fragmented landscape, both connectivity between habitat patches and increasing patch size can be crucial for species persistence by reducing the negative effects of demographic, environmental and genetic stochasticity on the viability of (meta)populations (Begon et al., 1996; Hanski and Gilpin, 1997; Harrison and Bruna, 1999; Ferreras, 2001; Petit et al., 2001; Frankham, 2005).

Genetic information on natural populations may help natural-resource managers to identify priorities and to predict the efficiency and consequences of their conservation management decisions (e.g. compensation or creation of habitat patches or corridors, conducting introductions or translocations of individuals). Firstly, neutral locus heterozygosity provides information on the genetic diversity within populations and thus population fitness and evolutionary potential (Reed and Frankham, 2003). Secondly, neutral genetic differentiation between populations and its correlation with geographical distance (i.e. isolation by distance) gives information on gene flow and helps us to understand how landscapes influence connectivity (Manel et al., 2003; Segelbacher et al., 2010). Finally, neutral genetic differentiation among populations is important to delineate possible evolutionarily significant units (ESU's) with different evolutionary potential (Crandall et al., 2000; Moritz, 2002; Manel et al., 2003). ESU's are reproductively isolated from other populations to a substantial degree, have unique or different adaptations

(Waples, 1991) and should merit separate management (Crandall et al., 2000). The potential ESU's should be taken into account in prioritizing conservation goals. For example, when several populations represent the same evolutionarily significant unit, conservation strategies that focus on the ecologically most intact populations would be more appropriate than attempting a near-impossible reversal of declining populations (Reynolds et al., 2011). In addition, invasive measures like reinforcement and reintroduction through translocations may disrupt the existence of ESU's (e.g. European wild rabbits: Delibes-Mateos et al. (2008) and black-lipped pearl oyster: Lemer and Planes (2012)).

One species that is a frequent subject of conservation concern is the natterjack toad (*Epidalea calamita*, formerly known as *Bufo calamita*). This endemic European toad is a typical pioneer species that breeds in shallow ponds in open landscapes like heathland and sand dunes (Beebee, 1979). Although natterjack toads are fairly common and widespread over much of south-west and central Europe, they have declined substantially at their range margins in northern France, Ireland, Britain, Belgium, Sweden and Estonia (Beebee, 2002). Natterjack toads are considered vulnerable under the Bern Convention of 1979 (Appendix II), and are listed on the EU Habitats and Species Directive of 1992 (Annex IV). On the IUCN Red List, the species is listed as 'least concern' with a decreasing population trend (Beja et al., 2009). Conservation measures for natterjack toad populations include protection and creation of permanent habitat patches and migration corridors as well as the creation of temporary habitats (Denton et al., 1997; Smit, 2006; Snep and Ottburg, 2008; McGrath et al., 2010). In addition, individual natterjack toads have been translocated when

83 their local habitat could not be saved, or reintroduced at suitable sites (The Netherlands:
84 Smit et al., 2006 and Britain: Denton et al., 1997; McGrath et al., 2010).

85 In Flanders (northern Belgium), the natterjack toad is present in all provinces, but
86 during the last decades many populations have disappeared due to habitat degradation and
87 fragmentation (Sparreboom, 1981; Stevens et al., 2004). Populations are currently limited
88 to the coastal dunes in the west and some metapopulations in heathlands in the north and
89 east, with some isolated populations under high anthropogenic pressure in the centre (e.g.
90 sandy urban development areas, clay pits and sand quarries), where the toads often cause
91 conflicts between nature conservation and urban development (Smit, 2006). Although the
92 natterjack toad receives a lot of attention from conservation policy makers, nature
93 organizations and landscape planners, little is known about the overall pattern of genetic
94 diversity, differentiation and gene flow of the inland natterjack toad populations in the
95 highly urbanized Flanders region. Genetic studies have been conducted in several parts of
96 Europe, for instance in populations separated by sea in Denmark (Allentoft et al., 2009),
97 mountainous areas in Spain (Oromi et al., 2012), coastal populations in Britain, (Rowe et
98 al., 1999; 2000b) and a coastal dune metapopulation in Belgium (Cox et al., 2017).
99 However, we expect that differences in habitat use as well as level of urbanization may
100 create variation in genetic population structure across the geographical range of the
101 natterjack toad. Therefore, care must be taken to extrapolate the results of these studies to
102 populations in Flanders.

103 The present study aims at describing the overall pattern of genetic diversity, genetic
104 differentiation and gene flow in several inland natterjack toad populations in Flanders. The
105 study populations represent a range of different landscape types and population structures,

106 from large (meta)populations containing several patches of suitable breeding habitat to
107 highly isolated populations in small habitat patches. We expected to find lower levels of
108 heterozygosity and high degrees of inbreeding in smaller populations with few breeding
109 ponds. Also, as Flanders is a heavily populated area with a dense network of roads and
110 industrial and urban land use, we expect high genetic differentiation and limited gene flow
111 among populations. Based on the results, we will discuss possible implications for the
112 conservation strategy of natterjack toad populations in Flanders.

Material and Methods

Study areas

Six natterjack toad populations in Flanders, Belgium were studied, which represent a wide range of population types in terms of number of habitat patches (assumed to be within dispersal distance) and surrounding land uses (figure 1). Two populations (Tielrode and Den Diel) consist of single breeding ponds surrounded by roads and buildings (Tielrode) or forest (Den Diel), and were considered to be strongly isolated from nearby populations (more than 10 km, following Smith and Green, 2005). A third study population (Liereman) is located in the nature reserve Liereman and is also isolated from nearby populations (at least 15 km), but surrounded by more permeable habitat like sand dunes and heathland. At the time of sampling, Liereman consisted of two subpopulations 700 m apart (each with one breeding pond) of which one was recently colonized: the sampling took place one year after the creation of this pond for conservation purposes. Two other populations (Kalmthout and Kikbeekbron) are part of large nature reserves: Border Park De Zoom (60 km²) and Hoge Kempen National Park (57 km²), respectively. Both consist of dozens of breeding sites with variable occupancy in recent years. In Kalmthout, three subpopulations were sampled (largest distance between subpopulations: 3.2 km and shortest distance: 0.7 km). In Kikbeekbron, we sampled adjacent breeding ponds with a maximum pairwise distance of 30 m, and these were considered as one subpopulation in the analysis. In both populations, breeding ponds were separated by sparsely vegetated heathland and areas with bare sand which probably favour re-colonization or reinforcement of subpopulations. Finally, three subpopulations (collectively called Linkeroever) were sampled in a highly disturbed area with landfills and recently created conservation ponds in the rapidly developing Left Bank section of the port of Antwerp. In this area, unused areas with pioneer biotopes have become highly suitable for natterjack toads. However, economic activities also result in a high turnover rate of those breeding patches (Snep and Ottburg, 2008). The largest distance between the three sampled subpopulations was 4.6 km and the shortest distance 1.5 km. All study populations were separated by at least 14 km. The largest distance between any two study populations was 73 km.

Sampling

Eggs were collected between 2008 and 2010 (for final sample size, see supplementary table 1). We visited the study sites daily or every two days, during rainy, warm periods throughout the breeding season. The

breeding season of natterjack toads consists of several breeding periods during spring and summer with low exchange of individuals between the periods, leading to distinct breeding cohorts within one season (Sinsch, 1988; 1997). The study of Sinsch demonstrated a higher genetic differentiation between these breeding peaks or 'breeding cohorts' than between nearby breeding ponds (but within the same breeding cohort). Possibly, this genetic differentiation between breeding cohorts is present in our study populations, therefore we sampled each (sub)population intensively throughout the whole breeding season covering all breeding peaks. Females in populations at the edge of the natterjack geographic range can lay a second clutch (Denton and Beebee, 1996). However, in central Europe, female natterjack toads generally lay one single undivided clutch every year (Sinsch et al., 1997) containing 3,000-4,000 eggs, and one clutch is generally fertilized by one single male (Banks, et al. 1994). To avoid sampling of full-sibs, we searched for distinct egg clutches before the embryos had hatched. The eggs were brought to the lab and put in aquaria in a common environment, and were reared until the larval stage, when stored at -80°C. DNA was extracted from the tail tissue. Since clutches can contain up to four different alleles per locus (two from female and two from male parent), strong variation could be observed between full siblings. Therefore, we included multiple eggs per egg strain in the analysis.

In the study populations Den Diel and Kikbeekbron, which were sampled in 2010, some clutches had hatched before collection. To avoid sampling bias in Kikbeekbron, we only considered larvae to be from different egg clutches when we collected them from separate, but adjacent ponds and/or from clearly different larval stages. In Den Diel, which consisted of one breeding pond, we collected as many larvae of different sizes as possible to avoid full-sibs, but discarded Den Diel from analyses where the parameter 'egg string' was included (see AMOVA).

DNA extraction and PCR conditions

DNA was extracted from the larval tail tissue using NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Qiagen Multiplex PCR Kit ® (Qiagen) was used to perform two multiplex PCR on microsatellite loci that were already developed for the natterjack toad (Rowe et al., 1997; 2000a, Rogell et al., 2005). Multiplex 1 contained primers for Bcalμ1, Bcalμ2, Bcalμ4, Bcalμ5, Bcalμ6, Bcalμ7, Bcalμ10, Bucap2 and Bucap3. Multiplex 2 contained primers for Bcalμ8, Bcalμ11 and Bucap5. All forward primers contained an

M13 tail sequence, following the economic M13-tailed microsatellite protocol of Schuelke (2000). Instead of labelling all forward primers, this tailed forward primer was used in combination with a universal fluorescently labelled M13 primer. The samples were genotyped using a 3130xl GENETIC ANALYSER (Life Technologies, Carlsbad, CA, USA) and alleles were scored with GENEMAPPER version 3.7 (Life Technologies). Four loci (Bcalμ10, Bcalμ8, Bcalμ11 and Bucapμ5) were discarded due to ambiguous or low scoring. In total, 184 samples from 99 different egg strings were genotyped.

Genetic analysis

Genetic variability in the six populations was assessed with GENEPOP version 4.0 at the population level (Rousset, 2008). This software was used to estimate observed (H_O) and expected (H_E) heterozygosity, to estimate the inbreeding coefficient (F_{IS}) and the frequency of null alleles, to test for linkage disequilibrium between pairs of loci in each population, and to test for deviations of genotype frequencies from Hardy-Weinberg equilibrium (HWE) expectations using an exact probability test. Following Chapuis and Estoup (2007), microsatellite loci that showed significant heterozygote deficiencies (HD's, that is, positive F_{IS}) in all populations were discarded from all analyses, because this would indicate null alleles, and the presence of null alleles may lead to overestimation of genetic differentiation. Consequently, two loci (Bcalμ1 and Bucapμ3) were discarded from further analyses because of high F_{IS} values across populations. Hence, we were able to use six polymorphic microsatellite loci for the genetic analysis in this study (Bcalμ2, Bcalμ4, Bcalμ5, Bcalμ6, Bcalμ7 and Bucapμ2).

Population genetic structure was investigated in several ways. First, isolation by distance was tested among all subpopulations by comparing a pairwise log-transformed geographic distance matrix to a pairwise genetic distance matrix with a Mantel test (1000 permutations) in GENEPOP version 4.0 (Rousset, 2008). Pairwise geographic distances were calculated with the DIST-function in R version 3.0.1 using Decimal Degree coordinates (R Core Team, 2017), and pairwise genetic distances were calculated as $F_{ST}/(1 - F_{ST})$ in GENEPOP version 4.0 (Rousset, 2008). Second, a hierarchical analysis of molecular variance (AMOVA), executed in ARLEQUIN version 3.11 (Excoffier et al., 2005), was used to partition genetic variation at three levels, namely, (1) among populations, (2) among egg strings within populations (nested in level 1) and (3) within egg strings (nested in level 2). We used the model for genotypic, codominant data with unknown

gametic phase, with deletion, transition and transversion weights equal to 1, Epsilon = 1×10^{-7} , and an allowed level of missing data of 0.05. The *P*-values of estimators were calculated by permuting alleles/genotypes within/between egg strings with 1023 resamplings. Because of the software's inability to use loci with too many missing data, and because Den Diel was excluded from this analyses because of the uncertainty about the siblingship, the analysis was restricted to the 146 samples of 79 egg strings for which all six microsatellites were scored successfully. ARLEQUIN was also used to test for genetic differentiation among all population pairs. The sequential Bonferroni's procedure was applied to the tests of linkage disequilibrium, F_{IS} and pairwise differentiation tests to correct for multiple testing (Rice, 1989).

Following Rowe and Beebee (2007), we used multiple methods for inferring population genetic structure. Therefore, we applied three methods, all based on Bayesian approaches to assess the most likely population structure. First, we used STRUCTURE version 2.3.4 (Falush et al., 2003). The number of clusters (*K*) was simulated from 1 to 10 with 20 replications. For all runs (1,000,000 MCMC repeats with a burn-in of 100,000), the admixture model was implemented, which assumes that individuals have derived ancestry from more than one population, as well as the correlated allele frequencies model, which calculates allele frequencies over all populations. STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt, 2012) was used to select the most likely number clusters, that is, *K* with the highest value of Delta *K*. For this *K*, the replication from the software STRUCTURE version 2.3.4 with the highest likelihood was selected for graphical presentation. For every individual, the proportion of the genome originating from each inferred population was computed (quantitative clustering method).

Secondly, we used the R-package Adegenet version 2.1.0 (Jombart, 2008; Jombart and Ahmed, 2011) in R version 3.3.3 (R Core Team, 2017). The number of prior clusters (*K*) that maximized the variation between clusters was determined with the clustering algorithm *k-means*. To identify the optimal number of clusters, *k-means* was run sequentially with increasing values of *K*. We tested values of *K* from 2 to 20. Different clustering solutions were compared using the Bayesian Information Criterion (BIC), and the optimal *K* was selected by visual inspection of the BIC-plot (indicated by an elbow in the curve of the BIC-plot). Subsequently, the *dapc* function was used to transform the data using Principal Component Analysis (PCA), and to conduct a Discriminant Analysis of the retained Principal Components (DAPC). Unlike *k-*

means, DAPC can benefit from not using too many Principal Components, in order to reduce over-fitting and instability in the membership probabilities (Jombart 2008). As such, DAPC was conducted with 12 PCs, which explained 90% of the variation. DAPC provided the F-statistic value for each of the PCs, and all axes with $F > 15$ were kept (of which the first two were used for graphical representation).

Third, we used BAPS 3.1 (Corander et al 2003) in both individual and group analysis modes (i.e. without or with prior information on the group in which the individual was sampled, respectively), with a simulated number of clusters (K) from 2 to 20. BAPS uses a stochastic optimization to infer the posterior mode of genetic structures, runs much faster than STRUCTURE (which uses Markov chain Monte Carlo algorithms) and does not require multiple trials for each possible value of K. It uses both genotype and, in group mode, sample group information to infer population clusters. In contrast to STRUCTURE in which each individual is attributed a proportion to each cluster, Adegenet and BAPS assign each individual to one cluster.

Results

In total, four natterjack toad populations were sampled in 2008 and two populations were sampled in 2010, generating 184 DNA samples from 99 egg strings (see supplementary table 1). The H_O , H_E and F_{IS} values and frequencies of null alleles per population–locus combination of the six microsatellite loci are given in table 1. The overall mean H_O across all loci for all populations was 0.43. None of the inbreeding coefficients remained significant after sequential Bonferroni correction (smallest P -value after Bonferroni correction $P = 0.0045$, $\alpha = 0.0016$ for Bcalu2 in Kalmthout). There were no significant deviations from HW expectations nor evidence for linkage disequilibrium.

There was no significant isolation by distance among subpopulations ($R^2 = 0.01$, $P = 0.36$). The AMOVA showed significant genetic differentiation ($P < 0.001$) at all investigated levels (table 2), namely, among populations ($\Phi_{ST} = 0.20$), among egg strings

within populations ($\Phi_{ST} = 0.15$) and within egg strings ($\Phi_{ST} = 0.06$). Pairwise Φ_{ST} among populations were all significant ($P < 0.001$), also after Bonferroni correction, with an average value of 0.20 (table 3). The Φ_{ST} was the highest between Kikbeekbron-Tielrode (0.28) and Kalmthout-Tielrode (0.24) and the lowest between Liereman-Linkeroever (0.14) and Liereman-Kalmthout (0.15).

Visual inspection of the Bayesian clustering analysis of STRUCTURE (delta K statistics) clearly identified 3 clusters (see supplementary figure 1). In Den Diel, Kalmthout and Liereman most individuals were assigned to the second and third cluster, whereas the first cluster was slightly better represented in Kikbeekbron and Linkeroever. In Tielrode, the largest proportion of individuals was assigned to the second cluster (figure 2). Overall, the proportions of individuals assigned to each cluster were symmetric, indicating the weak population structure. In Adegenet, the number of clusters (K) that maximized the variation between clusters from the *k-means* algorithm was 12 indicated by the initial sharp decline in BIC values up to $K = 12$ (see supplementary figure 2) of which PC1 ($F = 49.211$) and PC2 ($F = 32.539$) were plotted (figure 3). The 12 clusters resulted from a subdivision of each of the six study populations. Overlapping distributions of most of the clusters on the ordination plot indicated a low degree of genetic differentiation. Finally, BAPS analysis in the absence of sampling group information generated complex groups with very mixed constitutions (data not shown). This analysis proposed 14 clusters with a 0.93 probability (log likelihood of optimal partition: -1927.91; 12 and 13 clusters with a 0.03 and 0.04 probability, respectively). However, in group mode, BAPS discriminated 5 clusters with a probability of 1.00 (log likelihood of optimal partition: -2359.43), i.e. Kalmthout and Liereman were clustered, and all other populations belonged to separate clusters.

Discussion

Our analysis on the Φ_{ST} values revealed a significant differentiation among natterjack toad populations across Flanders, suggesting that these populations have been isolated with limited gene flow for some period of time. The results are in line with our expectations that the high anthropogenic pressure in the Flemish landscape disrupts natural dispersal between populations. Habitat fragmentation and habitat loss through urban development, roads and land-use change have probably increased the geographical distance as well as the degree of landscape resistance between populations, resulting in lower connectivity. Genetic differentiation among natterjack toad populations has previously been found in similarly fragmented populations that were also subjected to habitat fragmentation (Denmark: Allentoft et al., 2009 and insular populations in Sweden: Rogell et al., 2010). However, Marangoni (2006) found no genetic differentiation between southern Iberian populations separated by more than 100 km, as cited in Oromi et al. (2012).

The strong differentiation between populations based on Φ_{ST} was only weakly reflected in the structuring analyses. The clustering by STRUCTURE, Adegnet and BAPS showed several inconsistencies, which makes it difficult to draw definitive conclusions. Differences between the tests could be the result of the low number of loci analysed and the low sample size in some study populations. Other parameters could also be important to explain the results. For instance, postglacial colonization probably had an impact on the genetic structure of the current populations (Rowe and Beebe, 2007). Also, more recent changes in land-cover may have affected gene flow during the last centuries.

We found no indications of isolation by distance (IBD). No IBD was found in natterjack toad populations in Denmark as well, despite the strong genetic differentiation

396 (Allentoft et al., 2009). A panmictic population structure could have disrupted the IBD
397 pattern, however, this was clearly rejected in the Denmark study, and therefore Allentoft
398 et al. (2009) suggested that the absence of IBD reflected a lack of gene flow. This
399 explanation is however unlikely in our studied populations because Bayesian clustering
300 analyses revealed only a weak population structure in spite of the highly urbanized and
301 fragmented landscape in Flanders. The lack of IBD might also be explained by the use of
302 Euclidean distance without taking into account landscape elements that may act as barriers
303 or corridors. Yet, in our study, we expect Euclidean distance to be highly correlated with
304 effective distance (Euclidean distance modified for the effect of landscape and behaviour,
305 see Adriaensen, 2003). The subpopulations within the populations, which were considered
306 in the IBD analysis, were mostly connected by a landscape containing many sand dunes or
307 landfills which should offer low resistance for dispersal (Stevens et al., 2004). So, recent
308 gene flow between these subpopulations is highly probable. One of the two subpopulations
309 in Liereman had only been colonized one year before sampling, most likely from the other
310 subpopulation which is only 660 m away, and the only known breeding pond within the
311 maximum observed dispersal distance of natterjack toads (4411 m, Miaud et al., 2000).
312 Immediate founder effects through very recent colonization can increase the genetic
313 differentiation between the subpopulations and, in this way, influence isolation by distance.
314 However, recent founder events should also result in low H_O (Mayr, 1954) which is
315 contradicted by our results. Another possible explanation for the lack of isolation by
316 distance may be that gene flow is not distance-dependent. Cox et al. (2017) studied a
317 natterjack toad metapopulation near the Belgian coast and found that the two most distant
318 subpopulations were the least genetically differentiated. Rather than disperse over land,

toads or toad larvae may also disperse through aquatic routes (rivers), riparian areas, dried river beds (as found for several terrestrial vertebrates: Sánchez-Montoya et al., 2016), along beaches (Cox et al., 2017) or through dispersal of eggs by waterfowl. Although the latter is not confirmed in field-based studies on fish or amphibians, a great taxonomic range of invertebrates is found to be capable of being internally or externally transported by waterfowl (reviewed by Green and Figuerola, 2005; Frisch et al., 2007). In addition, toads may be accidentally dispersed through anthropogenic activities such as soil transport, as was evidenced by a radio-tracking study in the Netherlands (Van de Water and Zollinger, 2008). While little is known about such alternative dispersal mechanisms, the resulting long-distance dispersal could strongly disrupt patterns of IBD (e.g. Vanschoenwinkel et al., 2011). The same mechanisms could also have disrupted other signatures of population structure e.g. the weak clustering of the STRUCTURE, ADEGENET and BAPS analyses.

Our neutral genetic analyses showed overall high heterozygosity and no deviations from Hardy-Weinberg equilibrium. The measured heterozygosity is in line with a genetic study on natterjack toad populations in southern Belgium ($H_O = 0.33- 0.67$) (Stevens, 2006). A correlation between heterozygosity and individual fitness (i.e. larval performance) has been demonstrated in populations of natterjack toad (Rowe et al., 1999) and other amphibian species (e.g. European tree frog: Luquet et al., 2011). Larval performance in these studies included growth and development rate, which can impact adult survival (Altwegg and Reyer, 2003). Our results on the differentiation within and among egg strings within populations also suggest that there is considerable genetic diversity within the study populations.

Implications for conservation

Although heterozygosity is rather high, the significant genetic differentiation among our study populations suggests that current connectivity between populations is low (revealed by Φ_{ST}). Low connectivity increases the risks for both genetic and demographic stochasticity. According to Ottburg et al. (2007) the minimum viable population size for a network of natterjack toad populations with at least one core population is 500 breeding pairs. The sampled subpopulations on the Linkeroever belong to a network of sufficient size, according to Snep and Ottburg (2008). Although no standardized counts were made in the other sampled populations, some of them (Tielrode, Den Diel, Liereman) are suspected to be below the viability threshold based on observations during field work and in an online database (www.waarnemingen.be). Where possible, dispersal should be facilitated between natterjack toad populations through the creation of habitat patches acting as stepping stones and/or corridors. The latter will be especially valuable to bridge large gaps between populations but also more challenging because they require the creation of suitable conditions for both survival and reproduction. When roads form barriers between habitat patches which lie within dispersal distance, ecoducts or tunnels should be considered. In addition, the support of larger population sizes will reduce demographic stochasticity, risk of inbreeding and loss of genetic diversity (Frankham, 2005) and could offer a source of immigrants for re-colonization of small habitat patches (Naughton-Treves et al., 2003).

Whether or not natterjack toad populations are evolutionary significant units (ESU's) should be taken into account when conservation priorities and management actions are discussed. According to Robertson et al. (2015), an ESU is defined as a population that shows both neutral and adaptive genetic divergence . As a consequence,

crosses between individuals from genetically distinct populations can result in outbreeding depression, with offspring that are less adapted to their environment. Local adaptation in natterjack toad populations has been previously demonstrated in southern European (Gomez-Mestre and Tejedo, 2003). A common garden experiment with toadlets raised from eggs collected from 4 out of our 6 study populations (Liereman, Tielrode, Linkeroever and Kalmthout) suggested the presence of local adaptation (Maes et al., 2013) since higher locomotor performance was observed in toadlets from Kalmthout compared to the toadlets originating from Liereman and Tielrode. These results confirmed the hypothesis that ephemeral habitat patches in low-resistance matrix generally favour higher dispersal capacity, whereas loss and increasing isolation of patches can select against dispersal capacity. The significant Φ_{ST} values found in our study in combination with phenotypic differentiation in dispersal capacity found in the common garden experiment suggests that natterjack toad populations in Flanders may not act as a single ESU. The weak clustering by the Bayesian analyses, however, does not allow us to make strong conclusions on the exact delineation of ESU's. Nonetheless, our study clearly shows that unique evolutionary potential could be lost when one or more of our natterjack toad populations would go extinct, and that translocation of individuals to other existing populations should be considered with caution.

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560

561 **Tables**

562 **Table 1.** Summary of genetic variation in six populations of the natterjack toad (*Epidalea*

563 *calamita*) in Flanders, Belgium, for six polymorphic microsatellite loci.

	Den Diel	Kalmthout	Kikbeekbron	Liereman	Linkeroever	Tielrode
Samples (egg strings)	10 (10)	27 (14)	41 (11)	30 (21)	55 (37)	21 (6)
Bcalμ2						
amplification success	1.00	1.00	0.98	0.90	0.93	1.00
H_e	<0.01	0.27	<0.01	0.04	0.04	<0.01
H_o	<0.01	0.15	<0.01	0.04	<0.01	<0.01
F_{is}	-	0.45	-	<0.01	1.00	-
P	-	<0.01	-	-	0.01	-
Null alleles	-	0.18	-	<0.01	0.10	-
Bcalμ4						
amplification success	1.00	0.89	0.95	0.90	0.89	0.95
H_e	0.78	0.59	0.37	0.65	0.51	0.47
H_o	0.60	0.63	0.23	0.70	0.45	0.30
F_{is}	0.24	-0.07	0.38	-0.08	0.12	0.36
P	0.15	0.72	0.03	0.62	0.80	0.15
Null alleles	0.09	<0.01	0.11	<0.01	0.03	0.11
Bcalμ5						
amplification success	1.00	0.93	1.00	0.97	0.91	0.95
H_e	<0.01	0.52	0.20	0.64	0.43	0.47
H_o	<0.01	0.40	0.22	0.55	0.42	0.60
F_{is}	-	0.23	-0.10	0.15	0.02	-0.30
P	-	0.34	1.00	0.21	0.33	0.33
Null alleles	-	0.10	<0.01	0.05	0.18	<0.01
Bcalμ6						
amplification success	0.90	0.93	0.95	0.97	0.96	0.95
H_e	0.69	0.71	0.61	0.63	0.69	0.71
H_o	0.33	0.48	0.46	0.79	0.66	0.75
F_{is}	0.53	0.32	0.25	-0.27	0.05	-0.07
P	0.07	0.07	0.01	0.12	0.21	0.63
Null alleles	0.57	0.43	0.20	0.07	0.19	0.19
Bcalμ7						
amplification success	1.00	1.00	1.00	0.97	1.00	0.95
H_e	0.54	0.33	0.33	0.41	0.50	0.41

H_o	0.60	0.30	0.29	0.45	0.56	0.35
F_{is}	-0.11	0.10	0.13	-0.09	-0.12	0.15
P	1.00	0.48	0.29	0.77	0.07	0.59
Null alleles	<0.01	0.06	0.04	<0.01	<0.01	0.32
Bucap2						
amplification success	1.00	0.93	1.00	0.97	0.93	1.00
H_e	0.36	0.68	0.73	0.60	0.73	0.55
H_o	0.40	0.80	0.85	0.62	0.71	0.67
F_{is}	-0.13	-0.19	-0.17	-0.03	0.03	-0.23
P	1.00	0.28	0.09	0.22	0.56	0.40
Null alleles	<0.01	0.02	0.17	0.26	0.35	0.15

Abbreviations: F_{IS} , inbreeding coefficient; H_E , expected heterozygosity; H_O , observed heterozygosity; N , sample size (number of egg strings). Significant P -values for F_{IS} are given in bold; none remained significant after sequential Bonferroni correction. The frequencies of null alleles was tested for every population–locus combination.

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Table 2. Hierarchical AMOVA measured at three spatial scales of natterjack toad populations (*Epidalea calamita*) in Flanders, Belgium, for analyses of genetic structure. Levels included are (1) among populations, (2) among egg strings within populations (nested in level 1) and (3) within egg strings (nested in level 2).

Source of variation	Sum of squares	Variance components	Percentage of variation	Φ_{ST}	P
Among populations	30.07	0.09	6.20	0.20	< 0.01
Among egg strains within populations	141.12	0.20	13.75	0.15	< 0.01
Within egg strains	251.89	1.18	80.04	0.06	< 0.01

Abbreviation: AMOVA, analysis of molecular variance. The P -values of estimators were calculated by permuting alleles/genotypes within/between egg strings with 1023 resamplings

Table 3. Pairwise genetic distances (Φ_{ST}) between 5 study populations of natterjack toad (*Epidalea calamita*). Population Den Diel was excluded from this analyses because of the uncertainty about the siblingship. All Φ_{ST} values were found significant ($P < 0.01$).

	Kalmthout	Kikbeekbron	Liereman	Linkeroever
Kikbeekbron	0.231			
Liereman	0.152	0.213		
Linkeroever	0.181	0.162	0.143	
Tielrode	0.241	0.281	0.192	0.218

Figures

Figure 1. Sampling locations in Flanders, Belgium, of natterjack toad (*Epidalea calamita*) populations for analyses of genetic structure. The shaded UTM squares (5 x 5 km) show the range of the natterjack toad in Flanders based on field observations during the sampling period (2008-2010) accessible through the citizen science observation platform of the Flemish nature organization Natuurpunt (<http://www.waarnemingen.be>), and on the database of Hyla, the amphibian and reptile study group of Natuurpunt (<http://www.hylawerkgroep.be>). We included the UTM cells with more than one observation.

Figure 2. (a) The population structure with $K = 3$ as estimated by STRUCTURE with a Bayesian approach for the natterjack toad study populations in Flanders based on six polymorphic microsatellites. Each bar represents an individual toad and individuals are clustered per population (labels above each cluster). For graphics, POPHELPER Structure Web App (version 1.0.10) is used. (b) Pie charts based on the Q-values of the STRUCTURE output from the run with the highest likelihood presented as percentages of the three clusters for each of the study populations.

Figure 3. Ordination plot of the discriminant analysis of principal components (DAPC) generated by Adegnet for the 12 genetic clusters shown by the ellipses for all six study populations. The dots represent the individuals. The bottom-right inset shows eigenvalues of the principal components in relative magnitude

599 **Figure 1**

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Figure 2

b)